Biochem. J. (1986) 240, 593–596 (Printed in Great Britain)

The characterization of an acidic calmodulin-binding protein in brain cytoskeleton and membrane fractions

Paola STROCCHI and Jeffrey M. GILBERT*

Neurochemistry Laboratory, Laboratories for Psychiatric Research, Mailman Research Center, McLean Hospital, Belmont, MA 02178, and The Department of Psychiatry, Harvard Medical School, Boston, MA 02178, U.S.A.

One of the most abundant acidic proteins in rat brain has an M_r of 68000 and a pI of 5.6 (68K 5.6 protein) when analysed by two-dimensional gel electrophoresis. The 68K 5.6 protein was found in large relative amounts in brain cytoskeleton preparations and in membrane and supernatant fractions. High-salt washing and proteolytic digestion did not remove this protein from the membrane elements. The 68K 5.6 protein was also found in the microtubule-associated protein fraction of purified microtubules and was present in large relative amounts in preparations of intermediate-filament proteins. The 68K 5.6 protein binds to calmodulin in the presence of Ca^{2+} ions, and we found it to be an abundant acidic calmodulin-binding protein in brain tissue.

INTRODUCTION

Previous studies from our laboratory (Strocchi *et al.*, 1981) have identified a protein of M_r 68000 and pI 5.6 that is abundant in all subcellular fractions of brain tissue. Both neuronal and glial elements contain this protein (Strocchi *et al.*, 1984). In the rat, the 68K 5.6 protein is synthesized in the retinal ganglion cells and conveyed down the optic nerve in the slow phase of axonal transport; this protein is also synthesized by glia in the optic-nerve sheath (Strocchi *et al.*, 1984). The 68K 5.6 protein was also found to co-purify with microtubules and microtubule-associated proteins (Strocchi *et al.*, 1981; Whatley *et al.*, 1984). In studies of cell-free translation from brain polyribosomes, the 68K 5.6 protein was found to be synthesized exclusively on the free polyribosome population, as are the major cytoskeletal proteins, including tubulin subunits, actin and the neurofilament proteins (Strocchi *et al.*, 1982; Gilbert & Strocchi, 1983). Sobue *et al.* (1981) and others (Lee & Wolff, 1984) have identified a group of calmodulin-binding proteins in microtubule preparations and presented evidence that these proteins were tau factors. Many other proteins are known to interact with and bind to calmodulin, as previously summarized (Rasmussen & Barrett, 1984; Klee & Newton, 1985). The purpose of the present study was to characterize the 68K 5.6 protein further in terms of its association with cytoskeletal and membrane fractions and its binding to calmodulin.

METHODS

Preparation of subcellular fractions from rat forebrain

Cytosol and smooth-microsomal fractions were prepared from the rat forebrains as previously described (Strocchi *et al.*, 1981). Brain tissue was derived from 25-day-old male Sprague–Dawley rats obtained from Charles River Breeding Co. (North Andover, MA, U.S.A.). In some experiments the membrane fractions were incubated with trypsin and chymotrypsin in the presence of 3 mM-tetracaine, as described by Scheele *et al.* (1980). Protein was measured by the method of Sedmak & Grossberg (1977). Intermediate filaments were prepared from rat spinal cords by procedures for axon flotation described by DeVries *et al.* (1972) and for Triton X-100 disruption described by Liem *et al.* (1978) as modified by Brown *et al.* (1981).

Two-dimensional gel electrophoresis

This was carried out by a modification of the method of O'Farrell (1975) as previously described (Strocchi *et al.*, 1981). The lysis and isoelectric-focusing-gel buffers contained 6% Ampholines. The electrophoretograms were stained with Coomassie Brilliant Blue R250 (Schwarz-Mann, Spring Valley, NY, U.S.A.) or silver (Switzer *et al.*, 1979), and photographed.

Preparation of calmodulin- and phenothiazine-binding proteins

Forebrain tissue (2.0 g) from 30-day-old male Sprague–Dawley rats was homogenized in 10 ml of homogenization buffer (250 mM-sucrose, 50 mM-Tris/HCl, pH 7.0, 1 mM-$MgCl_2$, 0.5 mM-EDTA, 2.5 mM-dithiothreitol and 2.5 mM-$CaCl_2$). The homogenate was centrifuged at 10500 *g* for 10 min and the resulting supernatant re-centrifuged at 100000 *g* for 2 h. A 7.0 ml portion of supernatant (containing 8.4 mg of protein) was passed through a column containing 2.8 ml bed volume of calmodulin–agarose (Affi-Gel–calmodulin, from Bio-Rad Laboratories, Richmond, CA, U.S.A.) pre-equilibrated in homogenization buffer. The column was washed with 10 bed vol. of homogenization buffer and the calmodulin-binding proteins were removed with elution buffer (250 mM-sucrose, 50 mM-Tris/HCl, pH 7.0, 1 mM-$MgCl_2$, 0.5 mM-EDTA, 2.5 mM-dithiothreitol, 2.5 mM-EGTA). The elution fraction contained 360 μg of protein. In experiments to isolate calmodulin-binding proteins from membrane fractions, the homogenization buffer contained 0.4% (w/v) Triton X-100.

* To whom all correspondence should be addressed, at: Mailman Research Center, McLean Hospital, 115 Mill Street, Belmont, MA 02178, U.S.A.

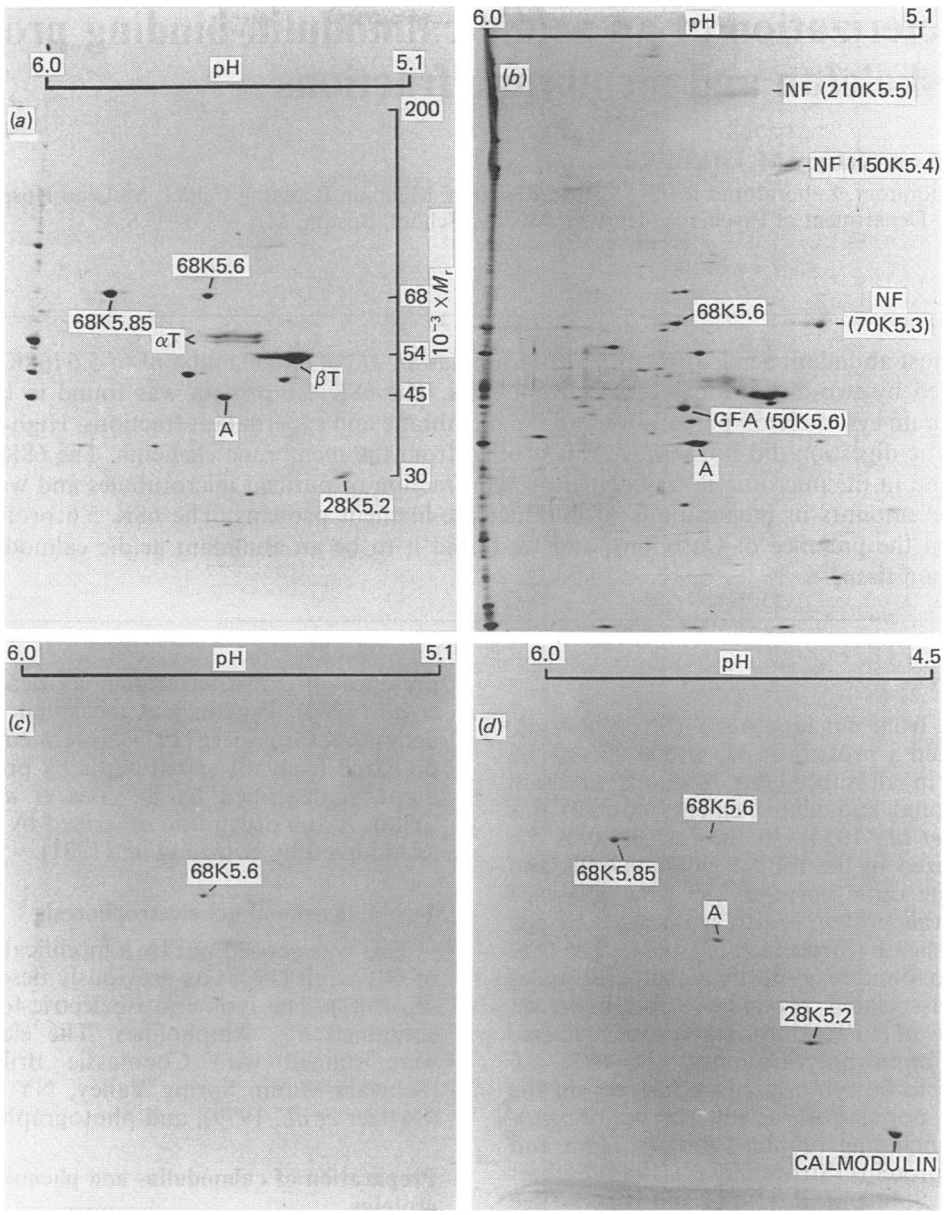

Fig. 1. Two-dimensional gel-electrophoretic analysis of proteins from rat forebrain tissue

(*a*) Analysis of supernatant proteins extracted from rat forebrain tissue. The position of the 68K 5.6 protein is shown relative to other proteins, including α-tubulin (αT), β-tubulin (βT) and actin (A). A 90 μg portion of protein was analysed, and the electrophoretogram was stained with Coomassie Blue and photographed. (*b*) Analysis of intermediate-filament proteins. The isoelectric-focusing gel was loaded with 35 μg of protein, and the electrophoretograms were stained with silver and photographed. (*c*) Analysis of calmodulin-binding proteins in the cytosol fraction of rat forebrain tissue. Calmodulin-binding proteins were prepared as described in the Methods section; 20 μg of protein was applied to the isoelectric-focusing gel. The electrophoretogram was stained with Coomassie Blue and photographed. (*d*) Analysis of phenothiazine-binding proteins in the cytosol fraction of rat forebrain tissue. Phenothiazine-binding proteins were prepared as described in the Methods section; 75 μg of protein was applied to the isoelectric-focusing gel. The electrophoretogram was stained with Coomassie Blue and photographed. The position of actin is denoted by A. Various proteins are denoted by '*x*K*y*', where *x* is the M_r ($\times 10^{-3}$) and *y* the pI. Other abbreviations: NF, neurofilament protein; GFA, glial fibrillary acidic protein.

In experiments to prepare phenothiazine-binding proteins (Levin & Weiss, 1976; Moore & Dedman, 1982), the rat forebrain tissue (2.0 g) was homogenized in 10 ml of homogenization buffer containing 300 mM-NaCl, 50 mM-Tris/HCl, pH 7.0, 1 mM-dithiothreitol, 0.2 mM-$CaCl_2$ and 0.1 mM-phenylmethanesulphonyl fluoride. The homogenate was centrifuged at 12000 *g* for 10 min and the supernatant re-centrifuged at 100000 *g* for 2 h. A 8.0 ml portion of supernatant (containing 9.1 mg of protein) was passed over a column containing 3.5 ml bed volume of phenothiazine–agarose (Affi-Gel–phenothiazine from Bio-Rad Laboratories) pre-equilibrated in homogenization buffer. The column was washed with 10 bed vol. of homogenization buffer, and the phenothiazine-binding proteins were removed with elution buffer (50 mM-Tris/HCl, pH 7.0, 5.0 mM-EGTA and 0.1 mM-phenylmethanesulphonyl fluoride). The elution fraction contained 828 μg of protein.

Detection of high-affinity Ca^{2+}-binding proteins on two-dimensional electrophoretograms

Acidic proteins in rat forebrain cytosol were separated by two-dimensional gel electrophoresis. The proteins were transferred to a nitrocellulose membrane by the method of Maruyama *et al.* (1984). The membrane was then incubated with $^{45}Ca^{2+}$ (as $CaCl_2$, obtained from ICN Chemicals, Irvine, CA, U.S.A.; sp. radioactivity 21.9 mCi/mg), and the protein–$^{45}Ca^{2+}$ complexes were detected by autoradiography as described by Maruyama *et al.* (1984).

RESULTS

Supernatant proteins from rat forebrain separated by two-dimensional gel electrophoresis show a major acidic protein at M_r 68000 with a pI of 5.6 (Fig. 1*a*). In previous studies from our laboratory (Strocchi *et al.*, 1981), this protein had also been found to be greatly enriched among the microtubule-associated proteins and to be present in various membrane fractions (Strocchi *et al.*, 1981), including a fraction enriched in smooth endoplasmic reticulum (Fig. 2*a*). The 68K 5.6 protein was not washed off the membrane fraction by high salt (0.5 M-KCl; results not shown) and was not totally degraded by proteolytic digestion (trypsin and chymotrypsin) as shown in Fig. 2(*b*). Therefore, it is unlikely that the 68K 5.6 protein is sticking to the membrane fragments as an artifact. Identical results were found in experiments with synaptic-membrane and rough-endoplasmic-reticulum fractions (results not shown).

As noted above, previous work (Strocchi *et al.*, 1981; Whatley *et al.*, 1984) showed that the microtubules contain relatively large amounts of the 68K 5.6 protein, which is purified by phosphocellulose chromatography in the microtubule-associated protein fraction. We also analysed proteins in intermediate-filament preparations by two-dimensional gel electrophoresis, as shown in Fig. 1(*b*). The 68K 5.6 protein was present in relatively large amounts. Its M_r is almost identical with that of the '70K' neurofilament protein, but it had a more basic pI, 5.6. The 68K 5.6 protein was also detectable in soluble and membrane fractions isolated from heart, liver and kidney tissue (results not shown).

Sobue *et al.* (1981) have described a group of low-M_r microtubule-associated proteins which bind calmodulin. We prepared soluble proteins from rat forebrain and purified these proteins by calmodulin affinity chromatography. Those proteins eluted with EGTA buffer (Ca^{2+}-free) were analysed by two-dimensional gel electrophoresis, as shown in Fig. 1(*c*). The 68K 5.6 protein was greatly enriched, being the major acidic calmodulin-binding protein found among the cytosolic fraction. The 68K 5.6 protein found in the smooth-endoplasmic-reticulum fraction could also be purified by calmodulin affinity chromatography in the presence of Triton X-100 (results not shown). As a control we subjected rat cytosolic proteins to phenothiazine affinity chromatography. Those proteins whose binding to phenothiazine was dependent on Ca^{2+} were eluted and analysed by two-dimensional gel electrophoresis, as shown in Fig. 1(*d*). Calmodulin, which is known to bind to phenothiazine (Levin & Weiss, 1976), was greatly enriched, along with several other proteins, including actin. Only trace amounts of the 68K 5.6 protein were present in the elution; this may have been secondary to binding of the 68K 5.6 protein to the calmodulin bound to the phenothiazine, and not direct binding to the phenothiazine. We also investigated the possibility that the 68K 5.6 protein was Ca^{2+}-binding. Brain cytosolic proteins were separated by two-dimensional gel electrophoresis and the

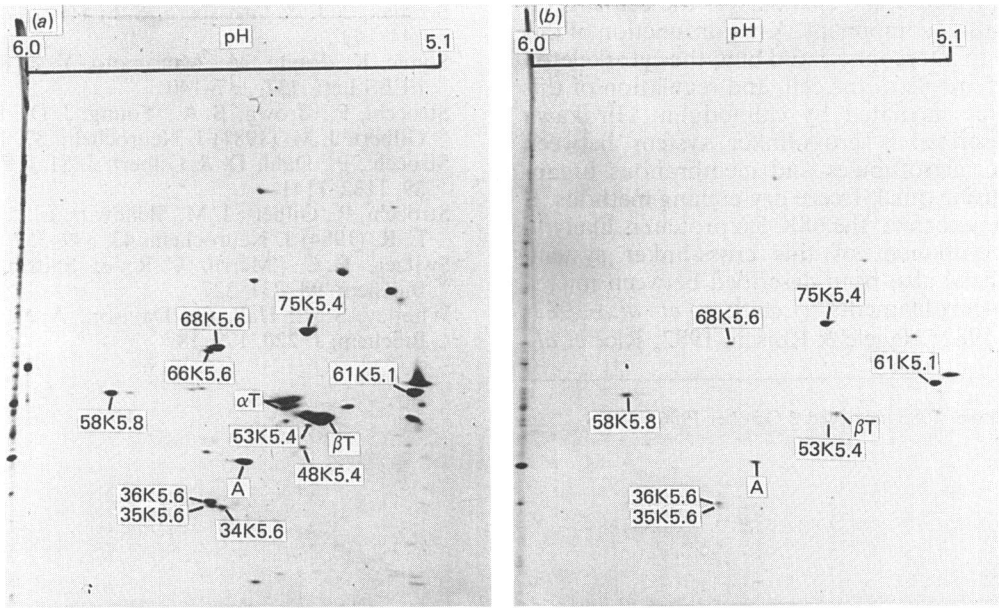

Fig. 2. Two-dimensional gel-electrophoretic analysis of smooth-microsomal proteins from rat forebrain before (*a*) and after (*b*) proteolytic digestion with trypsin and chymotrypsin

A 150 μg sample of protein was loaded on the isoelectric-focusing gel in (*a*) and 50 μg in (*b*). The electrophoretograms were stained with Coomassie Blue and photographed. Proteolytic digestion took place in the presence of 3 mM-tetracaine as described by Scheele *et al.* (1980). The notation is explained in Fig. 1 legend.

proteins were transferred from the electrophoretogram to nitrocellulose (Maruyama *et al.*, 1984). The nitrocellulose was incubated with $^{45}Ca^{2+}$ and washed. Calmodulin was the major Ca^{2+}-binding protein found in this experiment (results not shown); no acidic protein at 68K was seen to bind Ca^{2+}.

DISCUSSION

Our data show that there is one common protein, 68K 5.6, present in relatively large amounts in cytosol, membrane fractions (plasma membranes and smooth and rough endoplasmic reticulum) and cytoskeletal fractions (microtubules and intermediate filaments) isolated from rat brain tissue. The 68K 5.6 protein has been shown to be a microtubule-associated protein, on the basis of its purification properties, M_r, pI and peptide map (Strocchi *et al.*, 1981; Whatley *et al.*, 1984). Sobue *et al.* (1981) had identified a group of calmodulin-binding proteins of M_r 55000–62000 in microtubule preparations, and presented evidence that these proteins were tau factors. We have shown that the 68K 5.6 protein binds to calmodulin in the presence of Ca^{2+}; therefore, this protein may be identical with one of the proteins that Sobue *et al.* (1981) have described. However, Sobue *et al.* (1981) did not analyse their calmodulin-binding proteins by isoelectric focusing; therefore, we cannot be certain about the identity of the 68K 5.6 protein with the calmodulin-binding tau factors that they describe. Calmodulin is also known to interact with other cytoskeletal components, including tubulin dimers (Kumagai *et al.*, 1982), MAP2 (Lee & Wolff, 1984; Erneux *et al.*, 1984) and fodrin (Glenney *et al.*, 1982). There are many other calmodulin-binding proteins which are enzymes, and therefore present in relatively small amounts; these proteins would be undetectable in our gel system with the Coomassie Blue stain.

The relatively large amounts of the 68K 5.6 protein in cytoskeletal and membrane elements of the cell suggest that it is a structural component. A major function of this protein may be to interconnect and bind the cytoskeleton to membrane elements of the cell, and regulation of this function may be mediated by calmodulin. Hirokawa (1982) has described a cross-linker system between neurofilaments, microtubules and membranous organelles in axons, using quick-freeze dry-etching methods. If this conclusion is correct, the 68K 5.6 protein is likely to be a major component of this cross-linker system. Cross-bridges have also been described between microtubules and neurofilaments (Leterrier *et al.*, 1982; Minami *et al.*, 1982; Nagele & Roisen, 1982; Rice *et al.*, 1980), and the 68K 5.6 may also be a major component of these interconnections.

This work was supported by N.S.F. grant BNS-8416618 and the Marion Benton Trust.

REFERENCES

Brown, B. A., Nixon, R. A., Strocchi, P. & Marotta, C. A. (1981) J. Neurochem. **36**, 143–153
DeVries, G. H., Norton, W. T. & Raine, C. S. (1972) Science **175**, 1370–1372
Erneux, C., Passaveiro, H. & Nunez, J. (1984) FEBS Lett. **172**, 315–320
Gilbert, J. M. & Strocchi, P. (1983) J. Neurochem. **40**, 153–159
Glenney, J. R., Glenney, P. & Weber, K. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 4002–4005
Hirokawa, N. (1982) J. Cell Biol. **94**, 129–142
Klee, C. B. & Newton, D. L. (1985) in Control and Manipulation of Calcium Movement (Parratt, J. R., ed.), pp. 131–146, Raven Press, New York
Kumagai, H., Nishida, E. & Sakai, H. (1982) J. Biochem. (Tokyo) **91**, 1329–1336
Lee, Y. C. & Wolff, J. (1984) J. Biol. Chem. **259**, 1226–1230
Leterrier, J.-F., Liem, R. K. H. & Shelanski, M. L. (1982) J. Cell Biol. **95**, 982–986
Levin, R. M. & Weiss, B. (1976) Mol. Pharmacol. **12**, 581–589
Liem, R. K. H., Yen, S.-H., Solomon, G. D. & Shelanski, M. L. (1978) J. Cell Biol. **79**, 637–645
Maruyama, K., Mikawa, T. & Ebashi, S. (1984) J. Biochem. (Tokyo) **95**, 511–519
Minami, Y., Murofushi, H. & Sakai, H. (1982) J. Biochem. (Tokyo) **92**, 889–898
Moore, P. B. & Dedman, J. R. (1982) J. Biol. Chem. **257**, 9663–9667
Nagele, R. G. & Roisen, F. J. (1982) Brain Res. **253**, 31–37
O'Farrell, P. H. (1975) J. Biol. Chem. **254**, 7675–7683
Rasmussen, H. & Barrett, P. A. (1984) Physiol. Rev. **64**, 938–984
Rice, R. V., Roskensky, P. F., Pascoe, N. & Houghton, S. M. (1980) J. Ultrastruct. Res. **71**, 303–310
Scheele, G., Jacoby, R. & Carne, T. (1980) J. Cell Biol. **87**, 611–628
Sedmak, J. J. & Grossberg, S. E. (1977) Anal. Biochem. **79**, 544–552
Sobue, K., Fujita, M., Muramoto, Y. & Kakiuchi, S. (1981) FEBS Lett. **132**, 137–140
Strocchi, P., Brown, B. A., Young, J. D., Bonventre, J. A. & Gilbert, J. M. (1981) J. Neurochem. **37**, 1295–1307
Strocchi, P., Dahl, D. & Gilbert, J. M. (1982) J. Neurochem. **39**, 1132–1141
Strocchi, P., Gilbert, J. M., Benowitz, L. I., Dahl, D. & Lewis, E. R. (1984) J. Neurochem. **43**, 349–357
Switzer, R. C., Merril, C. R. & Shifrin, S. (1979) Anal. Biochem. **98**, 231–327
Whatley, S. A., Hall, C., Davison, A. N. & Lim, L. (1984) Biochem. J. **220**, 179–187

Received 22 September 1986; accepted 2 October 1986